bio-techne®

Application Note

Monitor Your mAb with Selectivity and Specificity for Upstream Bioprocessing

Simple Western™ Capillary Immunoassays for Biosimilar mAb Development

CE-SDS and cIEF Are Workhorse Tools for mAb Analysis but Require Purified Samples

Monoclonal antibodies (mAbs) represent many of the most profitable drugs sold, and the expiry of antibody patents presents an attractive opportunity to develop biosimilars. Biosimilars are identical in amino acid sequence to off-patent innovator products and promise comparable safety and efficacy. Drug development groups must thoroughly characterize critical quality attributes (CQAs) of innovator drugs, and biosimilars are compared to innovator products to ensure no clinically meaningful differences in safety, efficacy, and immunogenicity.

Antibodies are very complex compared to small-molecule drugs, and winning regulatory approval for innovator and biosimilar drug products can be challenging. Thus, there is a pressing need for advanced analytical tools to document CQAs that stem from the antibody's complex molecular structure, including size and charge heterogeneity imparted by post-translational modifications, fragmentation, and aggregation. Capillary electrophoresis platforms like CE-SDS and cIEF are standard and widely accepted methods for mAb characterization. However, users must first purify and concentrate antibody samples before analysis because proteins are detected directly by absorbance or native fluorescence.

Simple Western Provides Selective mAb Analysis in Unpurified Samples

Simple Western™ is a family of automated benchtop instruments that separate proteins by CE-SDS or cIEF followed seamlessly by immunodetection directly in the capillary. Get the resolving power of CE-SDS/cIEF with the sensitivity and specificity of ELISA for selective mAb analysis directly in complex samples like human serum.[1]

The Best of Both Worlds: Simple Western Combines Capillary Electrophoresis and Immunoassay Analysis

Combining CE-SDS/cIEF with antibody-based immunodetection is more sensitive than traditional CE-SDS/cIEF platforms with native fluorescence detection. Plus, Simple Western provides accurate, quantitative results you won't get with ELISA or Western blot, and results are ready in just 3 hours.

	ELISA	CE-SDS/cIEF	Simple Western
Immunodetection	Yes	No	Yes
Protein separation	No	Yes	Yes

A New Tool for Biosimilar mAb Development

Trastuzumab (tradename Herceptin®) is one of the earliest antibody-based therapies available for cancer treatment, and companies worldwide develop trastuzumab biosimilars. In this App Note, we describe a fast and easy Simple Western assay to characterize trastuzumab with results that are rich in quantitative detail. We show how protein separation on Simple Western uncovers cross-reactivity of the detection antibody with other IgG1 drug products, which may not be easy to identify on ELISA, and could produce misleading results.

Materials and Methods

All experiments were performed using the Jess™ Simple Western instrument. The materials used in this study are listed in TABLE 1. Samples were prepared as described in the 12-230 kDa Separation Module for reducing as well as non-reducing conditions (without DTT addition).

Item	Vendor	Part Number
Anti-Trastuzumab Antibody	Bio-Techne	MAB9547
Anti-Cetuximab Antibody		MAB9626
Anti-Rituximab Antibody		MAB9630
Anti-Mouse Detection Module		DM-002
12-230 kDa Separation Module		SM-W001
IdeS	Promega	V7511
Trastuzumab	Roche	N7283B01

TABLE 1. Materials used in this study. All primary detection antibodies are anti-idiotype antibodies. The anti-trastuzumab antibody was diluted to 1:500 for non-reduced samples and 1:250 for reduced samples.

Linearity

Linearity experiments were performed under reducing and non-reducing conditions at 5 different concentrations and at a fixed dilution of anti-trastuzumab antibody. The concentrations of reduced samples were 200, 100, 50, 25, and 12.5 µg/mL. The concentrations of non-reduced samples were 5, 2.5, 1.25, 0.6, and 0.3 µg/mL.

Intermolecular Specificity

The intermolecular specificity of the anti-trastuzumab antibody was tested by reactivity against different mAbs (cetuximab and rituximab) under reducing and non-reducing conditions. Reduced samples were evaluated at a final concentration of 50 µg/mL and non-reduced samples were tested at a final concentration of 12.5 µg/mL. Different anti-idiotype antibodies (anti-cetuximab and anti-rituximab, both diluted to 1:1,000) were checked against reduced trastuzumab with a final concentration of 200 µg/mL.

Intramolecular Specificity (IdeS Treatment)

The intramolecular specificity of the anti-trastuzumab antibody was tested on trastuzumab treated with IdeS to cleave at the lower hinge area. 4 µl trastuzumab (5 mg/ml) was mixed with 4 µl IdeS protease (50 unit/µl) and 40 µl buffer (250 mM sodium phosphate, 750 mM sodium chloride, pH 6.6). Then, the mixture was incubated at 37 °C for 1 hour and stored at -80 °C.

Results

To establish a Simple Western assay for trastuzumab analysis, we tested the ability of an anti-trastuzumab antibody to detect purified trastuzumab under reducing and non-reducing conditions. Under reducing conditions, a heavy chain peak emerged at 60 kDa a light chain signal peak appeared at 32 kDa (FIGURE 1A). Under non-reducing conditions, an intact IgG1 peak appeared at 151 kDa (FIGURE 1B). These molecular weights are consistent with those reported by SDS-PAGE analysis.[2]

Next, we sought to determine the linearity of the Simple Western assay for trastuzumab analysis under reducing and non-reducing conditions. A 2X serial dilution series of trastuzumab was prepared under reducing and non-reducing conditions and analyzed in duplicate. As expected, the trastuzumab signal decreased with decreasing sample concentration (FIGURE 2A,C). The average peak area was plotted against trastuzumab concentration, and a linear regression analysis was performed. This analysis revealed a highly linear detection range of trastuzumab under both reducing and non-reducing conditions (FIGURE 2B,D).

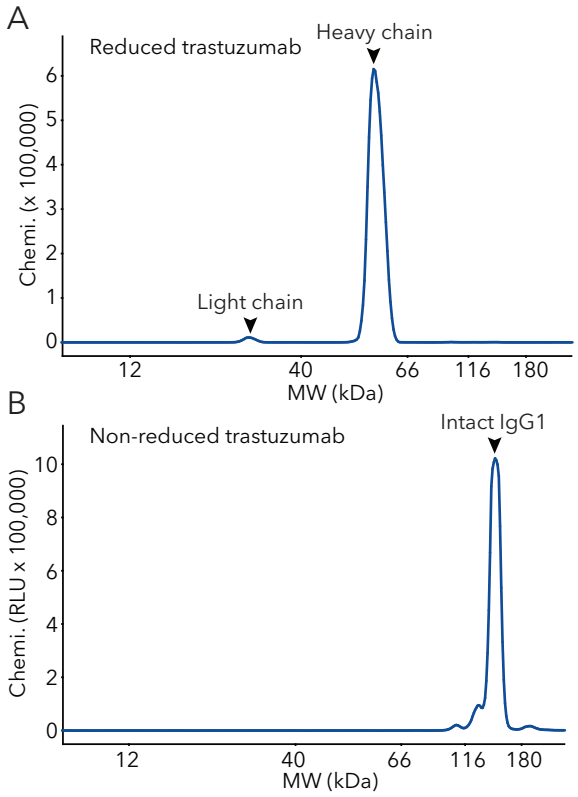

FIGURE 1. Simple Western analysis of trastuzumab. Trastuzumab was analyzed under (**A**) reducing and (**B**) non-reducing conditions using an anti-trastuzumab antibody.

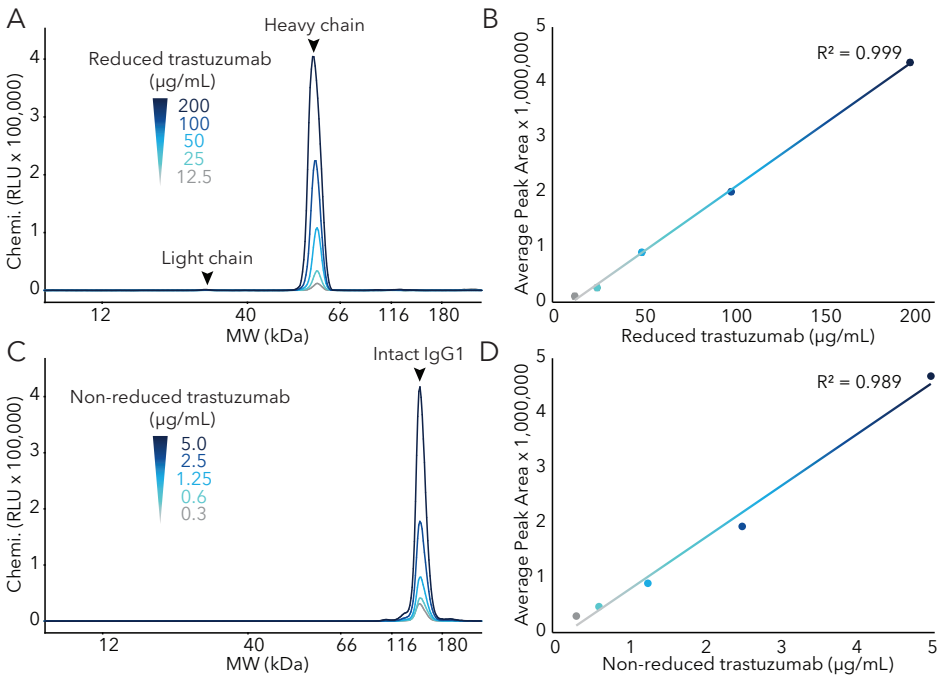

FIGURE 2. Linearity of trastuzumab analysis. Serial dilutions of purified trastuzumab were analyzed under reducing and non-reducing conditions on Jess using the anti-trastuzumab antibody. (**A**,**C**) Overlaid representative electropherograms resulting from each sample dilution. (**B**,**D**) The average peak areas (*n*=2) were quantified and plotted by trastuzumab concentration, and linear regression analysis was performed to generate a line of fit.

Reduced		Non-reduced	
Conc. (µg/mL)	CV (%)	Conc. (µg/mL)	CV (%)
200	7.8	5	3.9
100	7.3	2.5	0.8
50	4.9	1.25	7.0
25	7.8	0.6	6.4
12.5	3.2	0.3	4.9

TABLE 2. Reproducibility of trastuzumab analysis. Covariance (%CV) was calculated for each sample concentration (*n*=2).

Furthermore, the CV of each sample measured in duplicate was less than 8% (TABLE 2). The linear and reproducible detection of trastuzumab by Simple Western provides quantitative trastuzumab characterization that would be difficult to achieve with traditional Western blot analysis, which are only semi-quantitative.

To test the specificity of the anti-trastuzumab antibody, two additional antibodies from the IgG1 subclass, cetuximab and rituximab, were analyzed under the same reducing and non-reducing conditions. Interestingly, peaks corresponding to heavy and light chains also appeared in the cetuximab and rituximab samples, albeit with significantly lower signal intensities than the trastuzumab sample (FIGURE 3A). Likewise, a low-intensity peak was detected in the rituximab sample under non-reducing conditions at approximately the same molecular weight as intact trastuzumab (FIGURE 3B). We also tested if anti-cetuximab and anti-rituximab antibodies could detect trastuzumab. When reduced trastuzumab was analyzed at a constant concentration of 200 µg/mL, signals corresponding to the trastuzumab heavy chain were detected by the anti-cetuximab and anti-rituximab antibodies, though with significantly lower signal intensities than the heavy chain peak detected by the anti-trastuzumab antibody (FIGURE 3C).

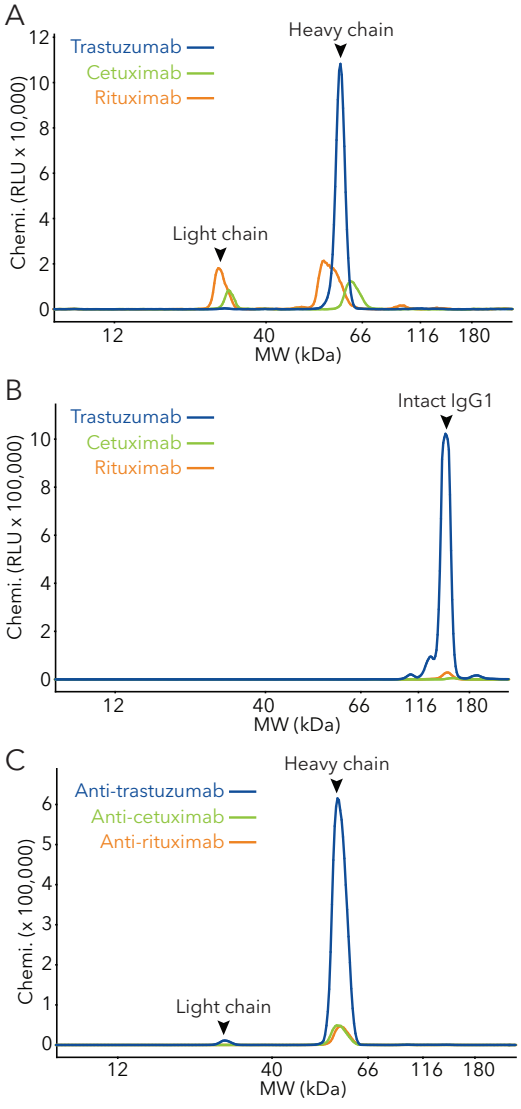

FIGURE 3. Intermolecular specificity of the Simple Western trastuzumab assay. (**A**) Reduced and (**B**) non-reduced samples of trastuzumab, cetuximab, and rituximab were probed with an anti-trastuzumab antibody. (**C**) Reduced trastuzumab was probed with anti-trastuzumab, anti-cetuximab, and anti-rituximab antibodies.

These results indicate that the anti-idiotypic antibodies used here have a low but detectable level of cross-reactivity with other monoclonal antibodies. Such cross-reactivity may not be readily detectable by ELISA and could produce inaccurate results. Thus, protein separation on Simple Western enables researchers to easily identify these instances of off-target antibody cross-reactivity.

Finally, we sought to understand the intramolecular specificity of the anti-trastuzumab antibody, an anti-idiotypic antibody expected to bind to the variable region. To do so, we treated trastuzumab with IdeS, a protease that cleaves at the lower hinger area of IgG1, and analyzed treated samples on Simple Western. Under reducing conditions, a Fab' peak appeared at 35 kDa (FIGURE 4A), and under non-reducing conditions, a F(ab')2 peak appeared at 101 kDa (FIGURE 4B). These results are consistent with previous IdeS digestion analysis of IgG1 by SDS-PAGE.[3] Therefore, the anti-trastuzumab antibody specifically binds to the variable region of trastuzumab, as expected.

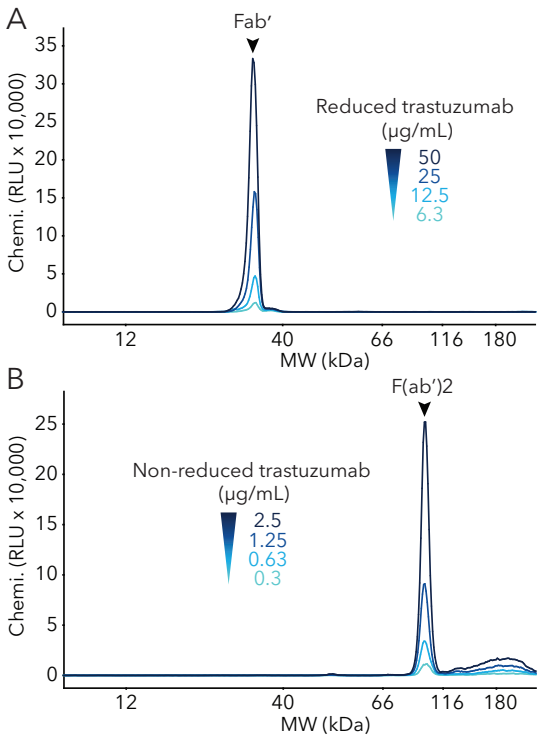

FIGURE 4. **Intramolecular specificity of the anti-trastuzumab antibody on Simple Western.** (**A**) Reduced and (**B**) non-reduced trastuzumab was treated with IdeS, a protease that cleaves IgG1 protein at the lower hinge area.

Concluding Remarks

Simple Western integrates the advantages of ELISA and CE-SDS/cIEF methods inside an automated benchtop instrument like Jess, creating a capillary immunoassay with fully quantitative reproducible results in just 3 hours.

- Unlike ELISA, Simple Western provides high-resolution protein separation to characterize size and charge heterogeneity of monoclonal antibodies, in addition to revealing any off-target cross-reactivity of the detection antibody. For more information on characterizing the charge heterogeneity of monoclonal antibodies with Simple Western, refer to our Application Note.[1]
- Unlike CE-SDS/cIEF, Simple Western uses antibodies for detection, which provides sensitive and specific detection. For more information on Simple Western analysis of mAbs in serum samples, refer to our Application Note.[1]
- Simple Western is an open platform, and any antibody may be used to characterize other monoclonal antibodies, biosimilars, and/or isoforms. Even lectins may be used to characterize antibody glycosylation. For more information on Simple Western glycan analysis, refer to our Protocol.[4]

References

1. Shining New Light on Pharmacokinetic Analysis with Simple Western, Application Note, ProteinSimple - a Bio-Techne Brand

2. Farràs M, Román R, Camps M, Miret J, Martínez Ó, Pujol X, Casablancas A, Cairó JJ. Heavy chain dimers stabilized by disulfide bonds are required to promote in vitro assembly of trastuzumab. BMC Mol Cell Biol. 2020 Jan 21;21(1):2.

3. Crivianu-Gaita V, Romaschin A, Thompson M. High efficiency reduction capability for the formation of Fab' antibody fragments from F(ab)2 units. Biochem Biophys Rep. 2015 Apr 25;2:23-28.

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